4 Considerations when Implementing a Genetic Diversity Assessment

Suggestions that genetic diversity should be used as an indicator of ecological health are not new (e.g., Beardmore *et al.*, 1980; Nevo *et al.*, 1988; Foré *et al.*, 1995a, 1995b). However, specific guidance on how genetic markers should be incorporated into an ecological assessment program is lacking. The overwhelming majority of reported scientific studies of genetic diversity are at relatively small scales, incorporating assays of at most a few dozen populations. Aspects of how to scale-up to region-wide analyses with dense geographic sampling, including data management and quality assurance issues, have not been considered. In addition, the focus of genetic diversity studies in the scientific literature is usually on the status of the species under study, not the ecosystem. The US EPA is currently engaged in several studies designed to evaluate the utility and practicality of implementing genetic diversity indicators as part of ecological assessment or monitoring. Based on our experiences with these studies and relevant information from the scientific literature, several guidelines are suggested.

4.1 Sampling Design

Two different types of sampling designs have traditionally been employed for ecological assessments: source-biased studies, in which sites with known exposures are compared to reference sites, and region-wide studies, in which a large number of sites are sampled according to a defined sampling scheme in order to create a regional profile. Some studies may have aspects of both designs, in which sites with known exposures are compared to a relatively large number of "reference" sites within the region. Both designs are amenable to genetic diversity analysis. Many examples of the source-biased design applied to genetic diversity exist in the ecotoxicological literature (see Table 2-1). In addition, there are many examples in the conservation genetics literature of genetic diversity analyses that incorporate regional scales, although they rarely include large numbers of sample sites within the region. To our knowledge, no examples yet exist of intensive regional ecological assessments that have utilized a genetic diversity indicator.

The source-biased design has obvious cost advantages when the assessment question of interest is whether a known, local exposure has an impact on the genetic diversity of resident populations. However, considerable care must be exercised when implementing this design. Because the intent of this design is to measure a recent genetic change, the reference population(s) will ideally be identical to the test population(s) in all aspects except for the application of the specific exposure, yet independent of the test population(s) following the exposure. Thus, the populations must have had similar genetic diversity before the exposure, either because they recently diverged or because they experienced high gene flow prior to the exposure. In addition, significant gene flow between the populations must have stopped immediately following the exposure and any genetic differentiation that occurred must have been due to the exposure and not to other population or environment-related factors. These standards are likely to be difficult to meet. In practice, genetic diversity is often measured at a number of reference sites and compared to the exposed site. If genetic diversity at the exposed site is outside the norm for the reference sites then the exposure is implicated as the cause of the change in genetic diversity. Here too, there can be difficulties with interpretation. Often, reference populations are chosen to be geographically distant from the exposed population in order to ensure that they represent "reference conditions". Typically, it is not clear that the reference populations are not each more closely related to each other than any is to the exposed population and that any genetic diversity differences uncovered did not predate the exposure.

Regional studies offer much greater ability to characterize patterns of intraspecific genetic diversity and their possible causes than do source-biased studies. Genetic diversity will naturally vary among popula-

tions for a variety of reasons, including variation in the size of populations that different habitats can support, as well as evolutionary relationships and patterns of dispersal among populations. These natural levels of genetic diversity are indicative of fundamental population data, such as effective population sizes and population boundaries (section 2.1). In addition, any attempt to determine whether anthropogenic factors have influenced present levels of genetic diversity must be able to distinguish historical (evolutionary) patterns from recent change. Since regional assessments allow better characterization of the natural variation in genetic diversity measures, they can provide guidance for selecting specific areas for more intensive study. For example, if genetic diversity of one population is determined to be low, it can be compared to evolutionarily similar populations to determine whether a recent genetic change is implicated.

The natural synergisms that genetic diversity data lend to landscape-level analyses and species assemblage studies suggest that incorporation of genetic diversity sampling into existing regional assessment programs, including EMAP and Regional EMAP programs, is the preferred strategy to obtain genetic diversity data at present.

The natural synergisms that genetic diversity data lend to landscape-level analyses and species assemblage studies suggest that incorporation of genetic diversity sampling into existing regional assessment programs, including EMAP and Regional EMAP programs, is the preferred strategy to obtain genetic diversity data at present. Sampling of target species for genetic diversity analyses can easily be accomplished within existing EMAP guidelines, and will help reduce costs of sample collection. One of the advantages of DNA-based analyses is that sufficient DNA can be obtained from a single fish fin-clip to perform a large number of genetic analyses. Thus, tissue can easily be collected in the course of normal field identification procedures and, in most cases, specimens can be released back into the environment.

In the long run, the most efficient method to measure anthropogenic changes in genetic diversity is to measure genetic changes directly through temporal monitoring. For this purpose, regional assessments provide an excellent means to collect the necessary baseline genetic data for future comparisons. In addition to regional-scale assessments, intensive temporal analyses of genetic changes at a network of index sites will be extremely valuable. Index sites typically are assessed with a greater range of diagnostic and condition indicators, which will allow for better integration of the genetic data. Since index sites are intended for long-term monitoring, they provide an excellent opportunity to measure the temporal scale of genetic changes. For both regional and index site studies, it will be critical that DNA or tissue is archived for future analyses as part of the assessment. Given the rapid changes occurring in molecular methodologies, it is likely that whatever marker is used to measure genetic diversity initially will not be the optimal strategy at later stages of the assessment. The availability of archived samples will allow future retrospective analyses to assess genetic changes using the most appropriate technologies available.

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Scale-up issues. Several project management issues emerge when the scale of genetic analysis changes from assessment of genetic structure at a few sites at a single point in time to assessment at regional scales and/or over time periods that may encompass decades. Clearly, the greater management complexity and cost of this type of project requires greater forethought in design of the genetic survey. The utility of a pilot study to guide project management cannot be overemphasized. The pilot study serves to validate the choice of molecular markers and species in the study region and allows for initial assessment of the feasibility of more intensive or large-scale sampling (Baverstock and Moritz, 1996). It is very likely that the project plan will be redefined based on the results of the pilot study. For example, a target species may be difficult to sample within the region or a number of molecular markers that were found to be useful in other areas may lack polymorphism (and thus discriminatory power) within the region. A pilot study also may determine whether the scale or intensity of sampling for a regional assessment is appropriate.

Management of a large genetic diversity survey will be logistically simpler if it can be divided into smaller units that are analyzed individually. For example, the EPA's pilot study of genetic diversity for central stonerollers throughout the Eastern Cornbelt Plains Ecoregion (section 3.1) was divided into analysis units that were equivalent to large watersheds or basins. In addition, analyses were completed for central stonerollers before attempting to assess genetic diversity in additional species. Geographical and species-stratified analysis has the additional advantage that fairly intensive genetic diversity assessments can be completed for specific basins on a regular basis throughout the life of the project. While the advantages of such compartmentalization of analyses are clear, it does lead to a potential for bias if variation in laboratory procedures occurs over time (thus, between sample units). Inclusion of duplicate samples from prior analyses as a type of positive control against temporal variation in laboratory procedures should control for this type of bias.

Additional scale-up issues involve planning for adequate data management structure and are dealt with in section 4.6.

4.2 Species Choice

Most genetic diversity studies in the scientific literature are focused on conservation or management of a particular species, usually one that is threatened, endangered, or of some economic importance. In choosing species as indicators of environmental health, other issues clearly come into play. Table 4-1 presents "optimal" species attributes for environmental monitoring. Many of these species attributes stem from consideration of basic population genetics. Genetic diversity of species that are highly sensitive to degraded habitat and that have short generation intervals will respond more quickly and more dramatically than other species. Species with low dispersal ability will have higher exposure to specific environments and may provide finer resolution of environmental differences between sites. Asexual species, including microbes and algae, are not optimal mainly because the distinction between intraspecific and interspecific genetic diversity is blurred. Valid use can be made of such species (e.g., Ford *et al.*, 1998) but they become functionally equivalent to species assemblage indicators.

Other species characteristics listed in Table 4-1 help make collection, analysis, and interpretation of the data simpler. Selection of species that are easily distinguished morphologically will ensure that genetic diversity is measured at the intraspecific level, and that comparisons are not erroneously made between different species (in fact, cryptic species complexes are readily diagnosed using molecular markers, which is one advantage of combining analysis of genetic diversity with species assemblage assessments). Selection of broadly distributed species allows simpler analysis of scale issues. Use of species that are important to resource managers will allow easier integration of genetic diversity monitoring into existing monitoring programs. Species that are moderately abundant within the study area are easier to

Table 4-1. Optimal characteristics of species assessed with a genetic diversity indicator.

Optimal Species Characteristics
• Short generation interval
• Moderate-high exposure to stressor(s)
• Moderate-high sensitivity to stressor(s)
• Low-moderate dispersal ability or highly philopatric
• Sexual reproduction
• Native species
• Broad distribution
• Moderate abundance
• Management importance
• Morphologically distinct
• Known life-history parameters (age structure, sex ratio, etc)
• Availability of comparative genetic and demographic data
• Availability of historical DNA or tissue samples
• Amenable to laboratory culture

collect, although these species are usually not management priorities. The population genetic structure of species that have not been excessively moved around is generally easier to interpret than the structure of introduced species. This is because the stocking history of introduced species is generally poorly known; non-native species could be useful indicators in cases where the history of introductions and their sources are well documented. Availability of historical data, including the availability of archival samples (DNA, fins, scales, or whole preserved specimens) is useful for reconstructing changes in genetic diversity that may have occurred prior to or during known exposures in the past. Finally, the selection of species that can be cultured in the laboratory will aid in assigning causality to genetic changes, if such studies are desired in the future.

Ideally, the indicator will be applied to several species, since a multi-species index should better predict ecosystem status than a single-species index. Genetic responses of individual species are not always predictable (Gillespie and Guttman, 1999), as different species "see" different aspects of the environment, and not always what we expect. If several species are selected then additional considerations become relevant, such as sampling from phylogenetically and ecologically diverse taxa.

4.3 Which Genetic Marker?

Several books have been published in recent years that review the biological and analytical properties of different molecular markers (e.g., Avise, 1994; Hillis *et al.*, 1996; Caetano-Anolles and Gresshoff, 1997). A general consensus is that no genetic marker is best for all applications and in the hands of all investigators; each provides different insights and requires different levels of investment in equipment and training. Here, we will summarize the properties of some of the most common genetic markers and discuss their relative strengths and weaknesses as ecological indicators.

Allozymes. Allozyme electrophoresis is a simple and time-honored technique in the field of population genetics. The principle of the technique is that allelic forms of enzyme proteins with different net charges will have different mobilities when induced to move through a matrix by application of an electric current (electrophoresis). Alternative forms of the enzyme at the enzyme locus (alleles) migrate different distances through the matrix and are visualized by histochemical staining (Figure 4-1). Numerous manuals have been developed that outline general equipments needs, procedures, and gel pattern (zymogram) interpretations (see May, 1992; Murphy *et al.*, 1996; and references therein).

Although allozymes are often thought of as "old technology", they have some clear advantages over other genetic markers as ecological indicators. Few other markers can match allozymes in the simplicity and economy of standard procedures. Allozymes have a much longer history than other genetic markers and have been analyzed in many more laboratories so the historical database of comparative population genetic data is much larger for this marker than for any other. In fact, it is highly likely that allozyme datasets can be found for any of the common stream fishes in the country. In comparison to some other markers, allozyme techniques suffer from a limited number of marker loci available for study and a limited number of alleles per locus. Less than a dozen polymorphic markers are assessed in typical studies, and most of these markers only segregate for two or three variant alleles. As noted earlier, allozyme loci are more likely to be affected by natural selection than most DNA markers, which may bias estimates of gene flow and genetic diversity. For example, allozyme markers suggested significant gene flow in oysters along the eastern and Gulf coasts of North America, but both mitochondrial and nuclear DNA markers indicated a sharp biogeographic boundary between northern and southern populations in northeastern Florida (reviewed in Avise, 1994).

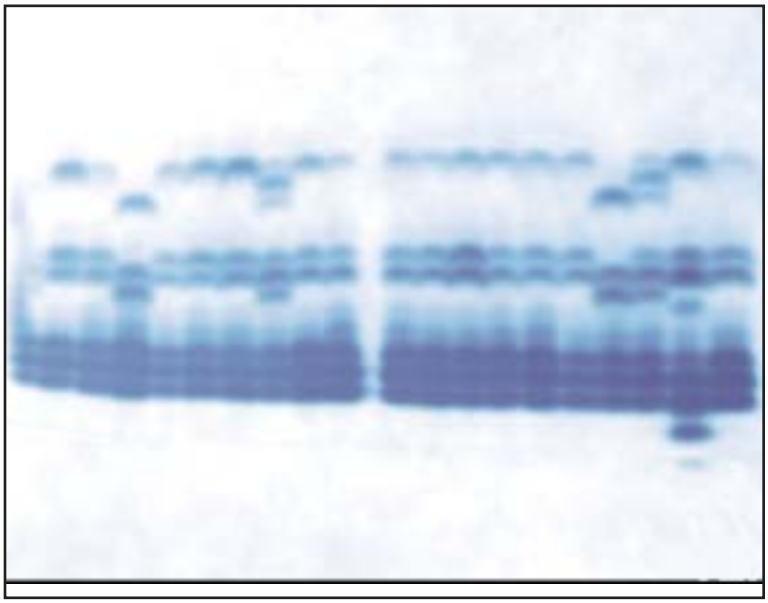

Figure 4-1. A histochemically stained starch gel showing GPI allozyme loci of rainbow trout. Each vertical lane represents a different individual.

Mitochondrial DNA Sequencing, RFLPs, and Prescreening Strategies.

Mitochondrial DNA is a circular DNA molecule containing about 16,500 base pairs that is present in multiple copies in the cells of eukaryotic organisms. Mitochondrial DNA has a number of properties that lend it to ecological assessments. Mutation rates tend to be higher than for most nuclear DNA regions, so large numbers of alleles (called haplotypes) are generated. Each individual usually only possesses one mitochondrial haplotype, which it typically inherits from its maternal parent. In addition, recombination within the mitochondrial genome appears to be rare or absent. Unlike different allozyme alleles, mitochondrial DNA sequences (Figure 4-2) can be analyzed to determine patterns of evolutionary relationships between different haplotypes. Thus, analysis of mitochondrial DNA sequences adds a different dimension to the analysis of genetic diversity; one can move beyond asking whether two individuals are the same or different at a genetic locus to ask *how* different they are at that locus. This provides a straightforward method for assessing (maternal) genealogical relationships between individuals of a population, and between individuals of different populations and different species.

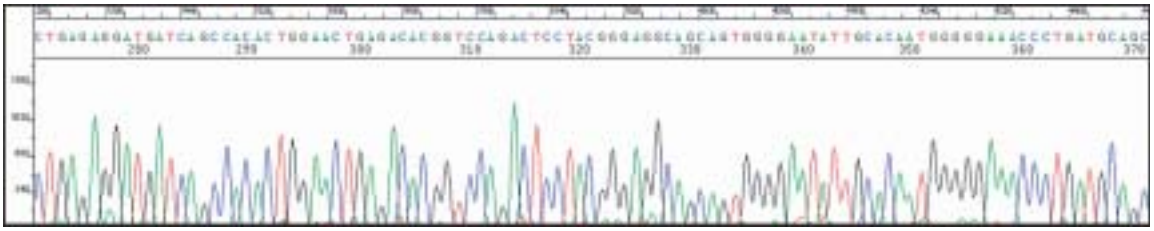

Figure 4-2. Electropherogram of DNA sequence generated by an automated genetic analyzer. Identification of mutational relationships among mitochondrial types (haplotypes) can elucidate patterns of evolutionary relationships among populations.

Mitochondrial DNA is not without drawbacks, the most important of which is that the lack of recombination within the molecule causes the entire mitochondrial genome to behave effectively as a single genetic marker; different mitochondrial genes are linked and therefore not independent. Since the history of a mitochondrial lineage is not identical to the population history (most notably, it says nothing about male contributions), interpretations made strictly from mitochondrial DNA may be erroneous.

The tremendous information content derived from DNA sequencing comes at a cost in terms of equipment, supplies, and technical expertise (Figure 4-3). Constant technological improvements are leading to rapid reductions in these costs. For example, the development of the polymerase chain reaction (PCR) and "universal" PCR primers has negated the need to isolate mitochondrial DNA away from nuclear DNA, or to clone specific fragments prior to sequencing (instead, the target sequence is simply PCR amplified). Meanwhile, a number of techniques have been developed that reduce the need for DNA sequencing or the number of required sequencing reactions. A common strategy is to screen for restriction fragment length polymorphisms (RFLPs). RFLPs provide a coarse indicator of DNA sequence variability, typically capturing less than one-eighth of the DNA sequence variability in a region. However, RFLP patterns contain information about relationships between haplotypes and can be analyzed relatively inexpensively. Other methods are used to "prescreen" mitochondrial DNA, so that only unique or previously uncharacterized haplotypes are sequenced. These include single strand conformation polymorphism analysis (SSCP), denaturing gradient gel electrophoresis (DGGE) and various commercial strategies. These techniques typically identify 80% to 100% of single-base mutations within DNA, but usually say little about haplotype relationships. Still, when combined with DNA sequencing, they can be more informative than RFLP analysis with only slightly more technical difficulty and cost (Figure 4-3).

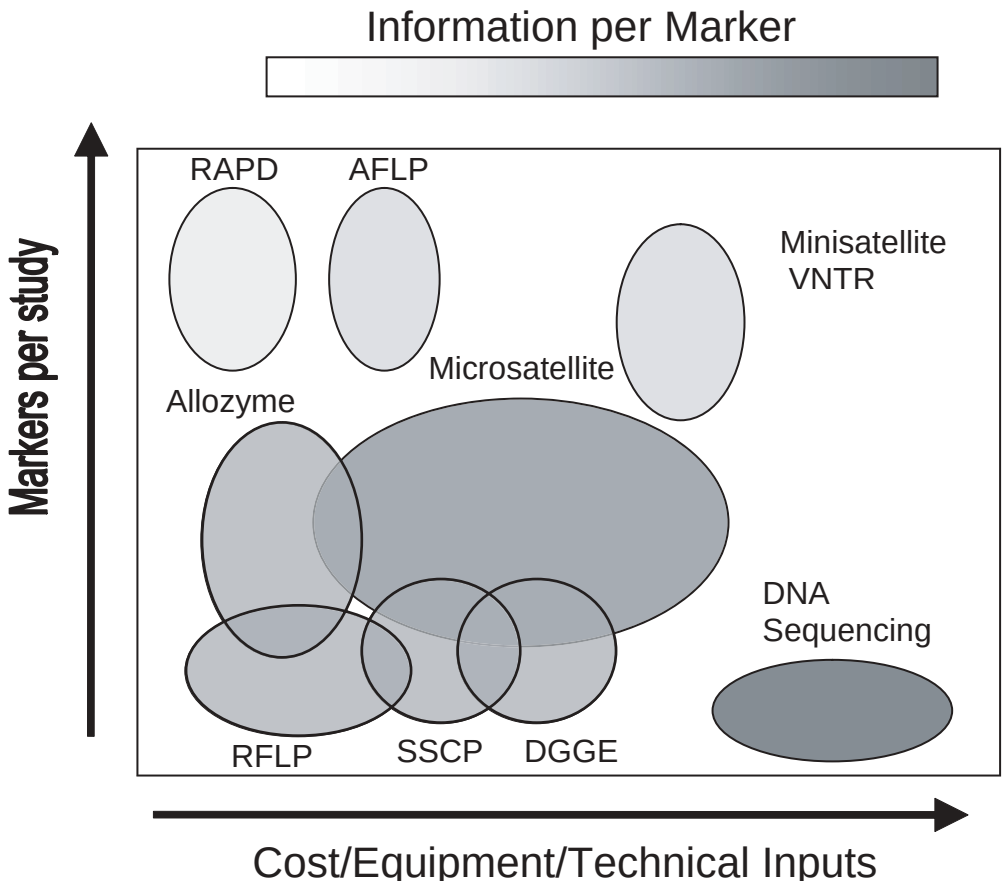

Figure 4-3. Relative advantages and disadvantages of different molecular marker strategies in relation to information per marker (darker colors in the figure being more informative), the number of markers per typical study, and costs per study in terms of capital outlay and technical expertise. The figure is not based on quantitative data and is presented for illustration purposes only.

Nuclear DNA Sequencing, RFLPs, and Prescreening Strategies. Strategies that are available for analysis of mitochondrial DNA also are available for the analysis of nuclear DNA genes, with similar advantages and limitations. The general strategy is often referred to as single-copy nuclear DNA (scnDNA) analysis. Typically, non-protein coding intervening sequences (introns) or flanking regions are targeted for analysis since they are usually more polymorphic than coding sequences. The primary advantage of scnDNA analysis is that many more genetic markers that are independent are available for analysis, so it can be highly complementary to mitochondrial DNA analysis. The development of gene sequence databases for many organisms, combined with PCR technology, has made available a number of well-studied genes for population analyses. A number of "universal" PCR primers have been published (e.g., Palumbi, 1996) to aid in the development of genetic markers for different species, although a certain amount of primer modification is often required. Because many of the gene sequences available for analysis are believed to have an impact on fitness, these, like allozymes, have the potential to be developed as diagnostic indicators of natural selection (and thus population stress). There are a number of disadvantages with scnDNA markers. In general, levels of polymorphism are low compared to mitochondrial DNA and some other nuclear DNA markers. In addition, the analysis is relatively intensive, even when using mutation-prescreening techniques, so relatively few scnDNA markers are generally analyzed per study.

Multi-locus DNA Fingerprints. DNA fingerprinting is a strategy that is in many respects the opposite of scnDNA analysis. Instead of targeting single, relatively well-characterized genes, DNA fingerprints target many anonymous chromosomal regions for analysis simultaneously. Typically, little is known about these regions except that they possess a small region of similarity to specific probes or PCR primers. Any region that has such homology presents as a distinct DNA fragment or "band" following gel electrophoresis. For any one individual, the pattern of presence and absence of bands can be highly complex, resembling a bar code. With the most variable markers, individuals can be uniquely identified by these band patterns. The most common DNA fingerprinting technique is random amplified polymorphic DNA (RAPD) analysis (Figure 4-4a), in which short primers of arbitrary sequence are used to amplify DNA fragments from 10-50 discrete regions in the genome. A newer method called amplified fragment length polymorphism (AFLP) analysis (Figure 4-4b) is similar, but depends on amplification of polymorphic fragments generated by restriction enzymes (RFLP) from arbitrary regions of the genome.

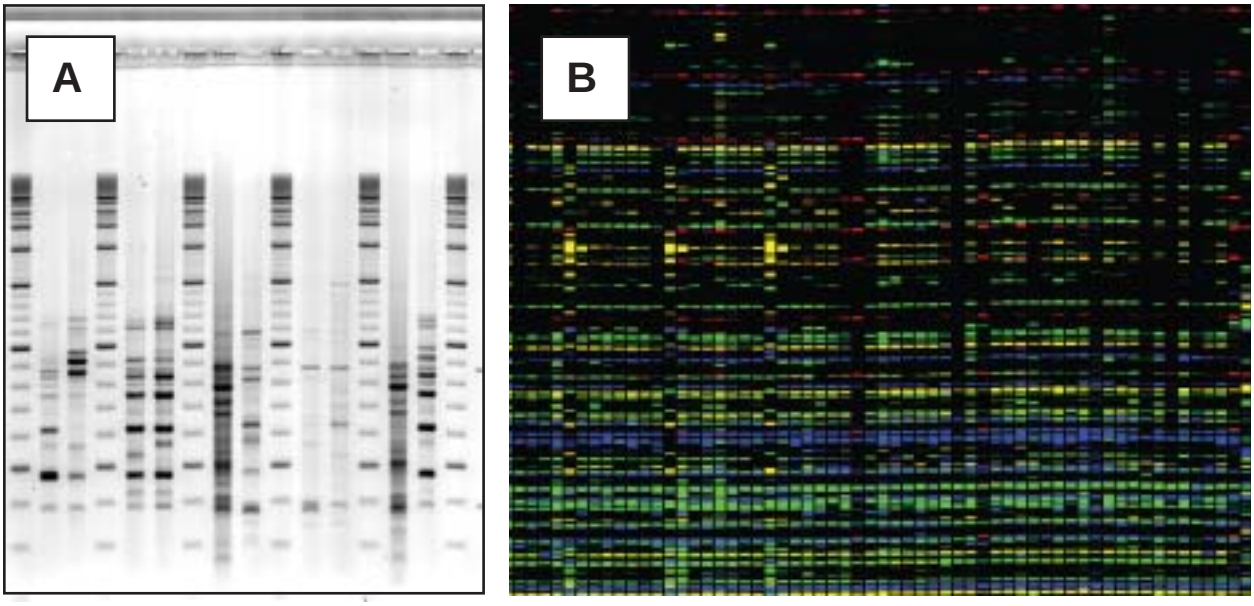

Figure 4-4. Examples of multilocus DNA fingerprints. Both RAPD (A) and AFLP (B) fragments can be generated without a priori knowledge of an organism's sequence. As dominant markers, homozygous and heterozygous individuals cannot be distinguished. The AFLP fingerprint here differs from Figure 3-14 because this is a multiplex AFLP reaction generated with an automated genetic analyzer.

There are two principal advantages of multilocus fingerprints for ecological assessments. The first is that no sequence information about the genome of the organism is needed in order to apply the methods. Thus, marker development costs are minimal and species can be chosen for analysis based on ecological or management criteria rather than the amount of sequence information already known. The second advantage is that genetic differences between individuals can be distinguished with relative economy. Dozens to hundreds of markers are analyzed in a typical DNA fingerprinting study.

The overriding disadvantage of DNA fingerprints is the poor quality of genetic information from each individual fingerprint band. Individual fingerprint bands cannot reliably be assigned to independent genetic loci. More importantly, RAPD and AFLP markers are dominant markers, which means that individuals that are heterozygous for a marker (i.e, only one chromosome of the pair has the marker) cannot be distinguished from individuals that are homozygous for the marker (both chromosomes have the marker), significantly decreasing the genetic information available. As a result of these ambiguities, comparisons are generally made in terms of overall 'similarities', taking into account the proportion of bands that are shared between individuals. Another concern with DNA fingerprinting methods is that sophisticated procedures must be implemented to reliably sort through the complex fingerprint patterns to identify homologous fingerprint bands from different individuals. Related to this is concern about the overall reliability of fingerprinting methods, particularly RAPD fingerprints. The RAPD technique is known to be very fickle, and adherence to exact protocols by different labs, often including use of the same brand of equipment and reagents, is considered critical to repeatability.

Microsatellite DNA Markers. Microsatellite DNA, also called simple sequence repeats (SSRs) are regions of repetitive DNA that consist of tandem repeats of a core sequence of two to five base pairs, such as (CA), (TAGA), and (CAT). Different alleles at a microsatellite locus differ in the number of tandem repeats of the core sequence. These sequences appear to be ubiquitous in the genomes of eukaryotes, and thousands of potential microsatellite markers could be developed for most species.

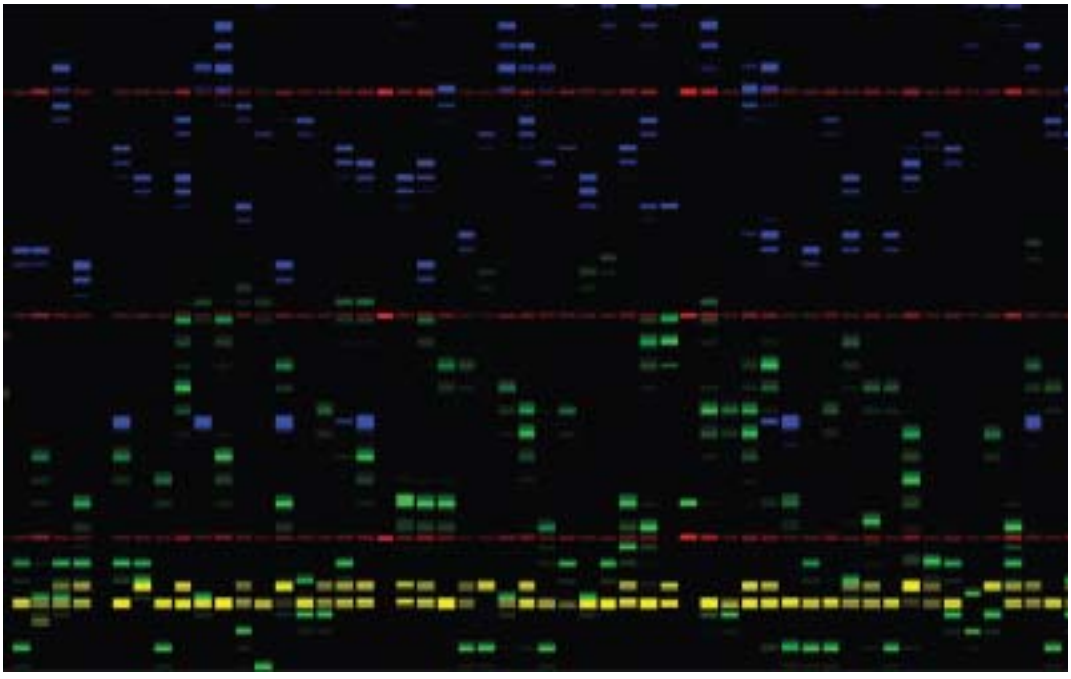

Figure 4-5. Flourescently labeled microsatellites detected using an automated genetic analyzer. Use of flourescently labeled markers allows differentiation between multiple loci (illustrated above by blue, green and yellow labeled markers) within the same reaction (multiplex PCR) thus reducing cost and increasing throughput.

Microsatellite DNA markers have some tremendous strengths for ecological assessments. They are subject to very high mutation rates relative to scnDNA, sometimes producing dozens of alleles (Figure 4-5). Like allozymes and scnDNA markers, inheritance of microsatellite markers is codominant, so heterozygotes can usually be reliably differentiated from homozygotes. The proportion of individuals that are heterozygous in a population is much higher than for other nuclear loci, often approaching 100%. Although heterozygosity at microsatellite loci is somewhat less sensitive to genetic bottlenecks than mitochondrial DNA diversity, it is much more sensitive than other nuclear DNA markers due to the large number of rare segregating alleles at these loci. Loss of these rare alleles actually provides a more sensitive measure of population bottlenecks than does heterozygosity (Leberg, 1992). It appears that the predominant mode of mutation is to an allele one repeat-unit different from the original allele [e.g., from $(CA)^{12}$ to $(CA)^{13}$], thus genealogical information can be captured from allelic relationships of microsatellite loci, although this information is less precise than that captured from DNA sequence analysis of mitochondrial DNA and nuclear genes. The primary disadvantage of microsatellite DNA markers is development cost. Technical expertise necessary for microsatellite marker development is greater than for any of the other markers listed, although, like DNA sequencing, the technical demands are decreasing. Microsatellite markers are developed from non-protein coding DNA regions and, therefore are not conserved across taxa, so that microsatellite DNA markers developed for one species are often only useful for very similar species. The number of organisms for which microsatellite markers have been developed is increasing rapidly, so it is possible that microsatellite development will be less costly in the near future. In addition, the very large number of alleles present at some microsatellite loci requires large sample sizes be used to estimate allele frequencies accurately.

Recommendations. This report has considered only a subset of the available genetic markers, but these are the most common and well supported in the scientific literature. The "best" marker for ecological assessments will vary, depending on the specifics of each situation. Until reliable methods are developed that allow economical analysis of nuclear DNA sequences from more than just a few genes per study, RFLP, SSCP, DGGE and sequence analysis of nuclear genes will likely be less informative than allozyme or microsatellite studies per unit effort. Mitochondrial DNA can be highly informative and offers insights not available from analysis of nuclear DNA. However, information from mitochondrial DNA may be misleading if interpreted alone so it is recommended that mitochondrial DNA be assessed only in conjunction with other markers.

Among the remaining markers, microsatellites undoubtedly offer the best combination of information per genetic marker and potential for analysis of many genetic markers. Although most microsatellite studies of natural populations to date have utilized relatively few microsatellite markers, there is now no technical reason why dozens, even hundreds of microsatellite markers cannot be developed and applied to genetic analyses. The technical challenges of microsatellite development can be overcome (for example, several commercial laboratories will now develop microsatellite markers on contract), however, an advanced laboratory is still required for microsatellite analysis. Reliable scoring of microsatellite markers requires the use of labeled PCR primers (either isotope or fluorescence) for visualization so, minimally, laboratories must have the ability to detect these labels. For large-scale, regional analyses utilizing many microsatellite markers, automated laboratory analysis using commercial genetic analyzers (automated sequencers) is essential.

The many advantages of allozymes, (economy, standardized methods, large existing database of information, homology of loci across species) should not be overlooked, particularly when microsatellite analysis is infeasible. Targeted analysis of specific allozyme loci (e.g., GPI) also may be useful as diagnostic indicators of specific stressors (e.g., heavy metals), and could complement analyses of other genetic markers. However, the requirements for ultra-cold storage of tissue samples in the lab and in

the field may make allozyme analysis impractical for regional analyses, particularly if the genetic collections are done as part of a multi-indicator assessment. In addition, if genetic diversity of allozymes is too low overall in the indicator species (as determined, perhaps, by a small pilot study), or if lethal sampling is not acceptable, then other methods should be explored. AFLPs and RAPDs, in that order, should be considered if neither allozyme nor microsatellite studies are feasible. Both methods allow assessment of many different genetic markers, but identification of AFLP markers is believed to be more repeatable between laboratories. However, AFLP analysis, like microsatellites, requires a more advanced laboratory able to detect isotopically or fluorescently labeled PCR products. Mitochondrial DNA analysis may be combined with any of the above nuclear markers to provide semi-independent genetic information. For example, analysis of evolutionary relationships among mitochondrial haplotypes may provide information on the historical biogeography of the species. Such information can help interpret patterns of genetic diversity in nuclear markers. A flow chart to aid in choosing genetic markers is provided in Figure 4-6.

4.4 Sample Size Considerations

Clearly, statistical power to detect differences in genetic diversity within populations and to detect genetic differentiation among populations will increase with increasing sample size. Two different sample sizes need to be considered: the number of individuals sampled per population and the number of markers assessed per individual. For any one marker, the ability to discriminate allele frequency differences between populations is low so large numbers of individuals need to be sampled from each population. In general, as more markers are assessed per individual, fewer individuals need to be sampled from each population. However, the balance between the number of markers and the number of individuals varies with the marker chosen. Recommendations for determining appropriate sample sizes are reviewed in current literature (Baverstock and Moritz, 1996).

In general, genetic markers that allow estimation of molecular relationships (DNA sequences, RFLP, and microsatellites) should require smaller sample sizes to achieve the same statistical power as markers that only consider allele frequencies, although this depends on the level and complexity of molecular relationships. Estimates of allele frequencies for microsatellite markers with high polymorphism (e.g., 10 or more alleles) can have very high standard errors since few individuals in the sample will possess any one allele.

For dominant markers (RAPD, AFLP) in which heterozygotes cannot be distinguished from one of the homozygotes, less information is available per locus and therefore more individuals or more markers must be sampled for the same statistical power as analysis of codominant loci. Often, logistical problems limit the number of individuals that can be sampled so statistical power is gained by sampling as many loci as possible. Simulation studies by Mariette *et al*. (1999) suggest that at least four times as many AFLP markers are needed as microsatellite markers to estimate genetic diversity within populations that are at equilibrium between migration and genetic drift. Comparatively more AFLP or RAPD markers will be needed to measure genetic diversity of populations that are not at equilibrium. Mitochondrial DNA, which is only transmitted from female parent to offspring (haploid inheritance), also has less genetic information than a single codominant marker, based on analysis of haplotype frequencies alone. Reduction in the standard error of estimates of haplotype frequencies can only be accomplished by increasing the number of individuals sampled.

Although it is difficult to come up with specific numbers, as a rule of thumb, sample sizes of between 50 and 100 samples per site are typically targeted in population genetics studies, although actual sample sizes are often lower. Analyses that utilize dominant marker systems should aim to assess between 100-

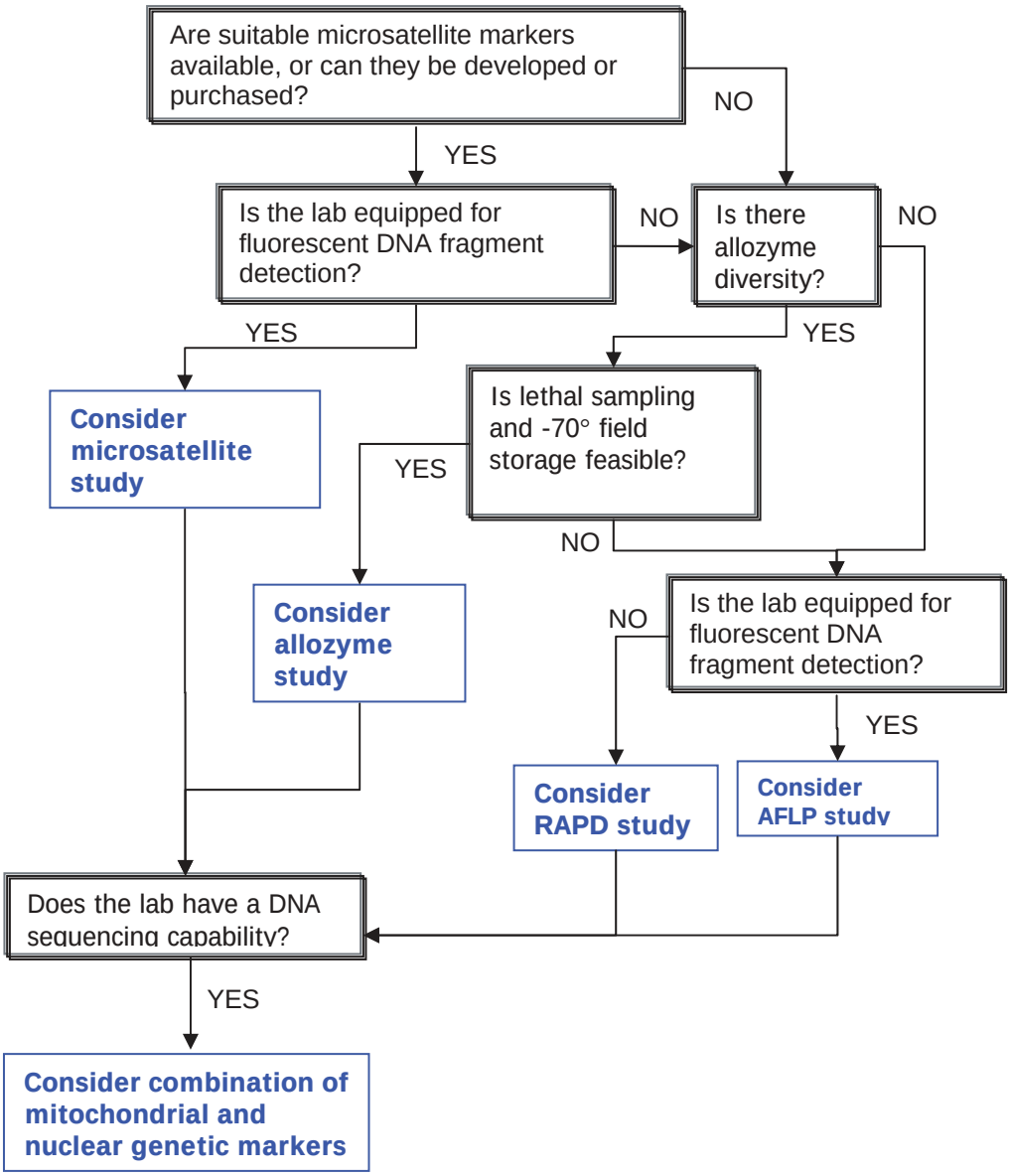

Figure 4-6. Flow diagram for deciding the best genetic marker or combination of genetic markers in relation to available resources.

200 polymorphic markers. Analyses that utilize codominant markers should aim for between 20 and 50 polymorphic loci.

4.5 Personnel Training and Specialized Equipment

Significant technical expertise is required for field sampling, laboratory analysis, and data analysis and interpretation. The degree of field expertise is equivalent to that needed for species assemblage indicators. Personnel must be able to operate sampling gear such as backpack electroshockers or seines effectively. In addition, they must have the ability to identify target species in the field. In practice, species discriminatory skills are probably less important than for assemblage indicators because the validity of the field identification will likely be confirmed by the genetic analysis.

Laboratory analyses require varying levels of skills. DNA extraction and quantitation techniques have become highly commercialized and many simple kits can be used that yield DNA of suitable quality and concentration for PCR-based analyses. The primary skill necessary is accurate pipetting. PCR analysis also is relatively straightforward and usually only requires good pipetting skills. However, a number of factors can disrupt the PCR process, and troubleshooting problems is a common occurrence in PCR assays. For this reason it is recommended that M.S.-level person with molecular biology training be on hand to supervise or consult during the PCR phase. A similar skill-level is recommended for gel preparation, sample loading and electrophoresis, and for operating automated DNA sequencers of genetic analyzers. Interpretation of gel patterns to determine the allelic complement at each marker analyzed generally requires a skilled, M.S.-level molecular biologist. In general, improvements in technology, such as highly robotic capillary electrophoresis systems for genetic analysis, have decreased the training requirements necessary to perform genetic analyses. Development of microsatellite markers requires sophisticated molecular biology skills.

Equipment requirements to perform different laboratory operations are described in Table 4-2. A number of software packages are available for genetic analysis, and similar analyses can be performed with general-purpose statistical software such as SAS©. Selection and interpretation of appropriate indices is best done in consultation with a population geneticist or a statistician who is familiar with genetic data.

4.6 Information Management

A typical genetic diversity study will generate large amounts of data. Critical data sets include the locations and dates of sampling, the number of individuals of each species that are sampled per site, and the genotype of each individual at each of the molecular markers analyzed. These data sets must be related to other databases that may exist, including phenotypic data (age, size, developmental abnormalities) biomarker data, chemical and physical habitat data, assemblage indicator data, and landscape-level data. This suggests that use of relational database software will be useful to manage the data, particularly if this software has already been incorporated to manage linked databases. However, simpler database management tools, including spreadsheets and simple database software, can be appropriate for less complex datasets.

Large numbers of tissue samples and DNA extractions will be collected that must be archived for validation purposes and to aid future retrospective assessments. Minimally, a database is needed that documents for each sample a unique sample ID, a population ID, the storage location of the tissue sample, the storage location of the DNA sample, dates of collection, DNA extraction, DNA quantification, as well as amounts and quality assessment of the archived material. If voucher specimens were collected along with the genetic samples then the database should include this link as well. Field data collection

forms and forms that document dates of laboratory manipulations and spatial orientations of samples during those manipulations (DNA extraction, PCR, electrophoresis, marker scoring) will need to be physically archived.

Metadata requirements include documentation of field collection procedures, as well as detailed laboratory methods (see appendices) and data analysis procedures. The procedure for translating genotypic data for an array of genetic markers into a data file must be explained, including descriptors of each of the fields in the data file. Similarly, documentation of the rationale and procedures for the statistical analyses, including software documentation, are needed.

4.7 Costs

Monetary costs of implementation of a genetic diversity indicator are incurred during sampling, laboratory analysis, and data analysis. Costs of field sampling for DNA analyses are similar to costs to collect species assemblage and biomarker data, and will generally be shared with those indicators. McCormick and Peck (2000) estimate the cost to field a contracted crew of 4 people at $1200 per site visited. They estimate the cost of field equipment, including a backpack electrofishing unit, to be $3515 per crew, with a 15% annual maintenance and depreciation rate. Sampling for allozyme analyses requires the use of special storage containers and a regular supply of dry ice or liquid nitrogen. This may add an additional $50 per site in supply costs, plus approximately $400 in cold storage equipment. One possible consequence of the necessity for cold storage is that the crew may not be able to remain at remote sites

Figure 4-7. DNA quantitation is performed using a commercially available fluorescent nucleic acid stain that is detected with a fluorescent scanner.

Figure 4-8. Thermal cyclers are used for fragment amplification (RAPD, AFLP, microsatellites) and DNA sequencing assays.

Figure 4-9. A capillary-based, auto-loading genetic analyzer can perform both fragment analysis (AFLP, microsatellite) and DNA sequencing.

Table 4-2. List of standard and specialized equipment for different types of genetic marker studies. * optimal but not necessary.

All laboratories

1. Freezer- samples
2. Freezer- chemicals
3. Refrigerator
4. UV transilluminator
5. Ultrapure water source
6. Pipetters
7. Variable speed centrifuges
8. Microcomputer with statistical genetics analysis software

Standard Equipment - DNA laboratories

1. Separate sample preparation room/area (DNA extraction)
2. PCR room/area
3. Post-PCR room/area with photodocumentation and/or fragment analysis equipment
4. thermal cyclers
5. Agarose gel electrophoresis rigs
6. Microcentrifuges (10,000 RPM)

Specialized equipment- allozymes

1. -80 freezers
2. dry ice or liquid nitrogen and canisters
3. Starch gel electrophoresis rigs
4. Power supplies (to 350 V, 150mA)
5. Incubator oven.
6. Chiller (for cooling starch gels during run)*

Specialized Equipment - microsatellites

Microsatellite Development

1. Hybridization oven
2. Incubator oven
3. Automated DNA sequencer
4. Shaking incubator
5. Fluorescence detection system (see microsatellite screening equipment)

Microsatellite Screening

1. Fluorescence detection system, either
   (a) Acrylamide gel electrophoresis rigs, 1000 V power supply, fluorescence scanner, fragment analysis software, microcomputer
   (b) Automated DNA sequencer with fragment analysis software

Specialized Equipment - RAPD

1. Power supplies (to 350 V, 150 mA)
2. Specialized agarose gel electrophoresis rigs for recirculating buffer
3. Chiller unit (to 0º C) for cooling agarose gels during run*
4. Microcomputer with fragment analysis software

Specialized Equipment - AFLP

1. Fluorescence detection system, either
   (a) acrylamide gel electrophoresis rigs, 1000 V power supply, fluorescence scanner, or fragment analysis software, microcomputer
   (b) Automated DNA sequencer with fragment analysis software

long before samples must be shipped to the laboratory, which may increase sampling costs.

Laboratory costs are more variable, and depend greatly on both the type of molecular marker assessed and the technical skills of laboratory personnel. All estimates given here are provided with the caveat that they are likely to change rapidly. As with field sampling, labor is the greatest cost. Based on review of the EPA's pilot study that utilized the RAPD fingerprinting technique, it is estimated that a crew of four laboratory technicians can extract DNA from 95 samples, perform nine RAPD PCR assays per sample, electrophorese each PCR product individually, and size each of the fingerprint bands in a period of approximately 9 days. Assuming a typical sample size of 50-100 individuals per site, this indicates that a crew of four will require between one and two weeks to assay a single site. Supply costs, including enzymes, agarose, plastics and chemicals, are estimated at $9 per sample for nine RAPD assays. Equipment costs included two fluorimagers and associated computer hardware and software ($70,000 each) ten agarose gel units ($4000), chiller ($3000), and many smaller items (pipetters, stirrers, centrifuges, incubators, approximately $10,000). We assume an average depreciation of approximately 20%. The fluorimagers could reasonably be replaced with cheaper photodocumentation systems for less than $20,000.

Guidance on costs of other types of molecular marker studies can be obtained from estimates by commercial laboratories. It is assumed that this is a maximum cost estimate because a profit margin is built in, but depreciation costs are built into the estimates and these labs may experience higher efficiency than other labs. Allozyme electrophoresis typically costs between $10 and $40 per sample, with between 5 and 15 polymorphic loci (and often many more monomorphic loci) assayed. Costs to set up an allozyme laboratory fall in the range of $10,000 to $20,000. DNA sequencing typically costs $25-$40 per sample, for a sequence of up to 500 bp. It is often recommended that sequences be generated for both the upper and lower DNA strands, doubling the cost. This cost does not include the cost of DNA extraction and PCR amplification of the target locus, approximately $2 per sample. Automated DNA sequencers vary greatly in price and quality, but useful models can be obtained for between $70,000 and $130,000. Microsatellites and AFLP fingerprints that are assayed on automated DNA sequencer cost between $2 and $5 per sample run. For microsatellites, a single automated run may include between one and 8 distinct PCR reactions, increasing the efficiency of the assay. Costs of DNA extraction ($2) are not included.

Costs of marker development range from essentially zero for RAPD, AFLP, and allozymes to several thousand dollars for microsatellites. At least one commercial laboratory will guarantee production of ten usable microsatellite markers for any species for a cost of $20,000.

4.8 Summary Recommendations

Among the myriad different ecological indicators available for environmental assessments, the optimal niche of a genetic diversity indicator is to map patterns of population structure and to identify cumulative genetic changes in populations through spatial and/or temporal comparisons. Like other indicators of ecological condition, its value will be greatly enhanced if it is interpreted as part of a multi-indicator assessment. By its nature, genetic diversity is a generalized indicator of long-term changes in populations; it will be difficult to assign causation to any specific stressor. There are exceptions to this rule. For example, assays for specific allozyme genotypes may be developed as diagnostic indicators of particular classes of stressors (e.g., heavy metals) and, as more is learned about functional consequences of nucleotide variation at specific genes, DNA-level diagnostic indicators also may be developed. Presently, however, a genetic diversity indicator will be most useful as an integrative indicator of genetic effects imposed by multiple stressors. This suggests that the genetic diversity indicator will prove most

useful as one component of a multi-indicator regional or index-site assessment.

Although the point can be debated, of the currently available technology, microsatellite analysis, perhaps combined with mitochondrial DNA analysis, is likely to provide the most useful information per unit effort for both regional genetic diversity assessments and temporal genetic diversity monitoring. The US EPA's Molecular Ecology Research Branch employs this methodology for most current and planned assessments. Unfortunately, this strategy also requires relatively advanced instrumentation and technical expertise compared to other strategies. The EPA's ECBP pilot genetic diversity study (section 3.1) was conceived and implemented to use RAPD fingerprinting precisely because of concerns about the ability to transfer genetic diversity indicator technologies to end-users. Because RAPD fingerprinting is technically simple, it was assumed that it would have the greatest prospects for technology transfer. However, during the course of the pilot study it became clear that the trade-off that comes with ease of implementation is that of extreme sensitivity to minor variations in laboratory techniques. Similar concerns are echoed in the scientific literature (see Perez et al., 1997). Concerns about repeatability between different laboratories appear to be great enough to negate any perceived technology transfer advantages. Current work with AFLP fingerprints indicates much less concern with repeatability, but technology transfer is considerably more difficult than for RAPD analysis. Transfer of allozyme technology should not be difficult, but the needs for lethal sampling and methods to transport samples at ultra-cold temperatures limit its general application.

While complete protocols for development and analysis of microsatellite markers may be difficult to transfer to environmental labs, certain aspects of the analysis are relatively straightforward. As mentioned, methods for extracting DNA from animal tissues have been commercialized and are now sold by several vendors as kits. The quality and quantity of DNA extracted using commercial kits is typically very high. In addition, improvements in thermal cycler technology, together with packaging of PCR reagents as standard assay kits have greatly eased technology transfer of PCR. Several vendors now sell test kits for genotyping domestic animals and humans with fluorescent microsatellite DNA markers. This suggests that regional field labs could accomplish major parts of the genetic diversity analysis while one or two "core" molecular biology labs handle other aspects. A model for this approach is shown in Figure 4-10. The regional lab, perhaps with some advice from a genetic analysis lab, would design the assessment, which presumably will be performed in conjunction with other ecological indicators. A marker development laboratory can then be employed to develop a panel of markers that are appropriate to the assessment. The regional lab can then collect samples and prepare DNA for analysis. The actual PCR reactions can be performed by the regional lab or by the genetic analysis lab. The genetic analysis lab performs the genetic analysis and derives the genetic diversity interpretation. The regional and genetic analysis labs can then assess the ecological significance of the genetic diversity indicator data.

Under this scenario, the marker development laboratory could be a commercial laboratory or a laboratory internal to the EPA. The EPA currently has the required expertise for such a laboratory in the Molecular Ecology Research Branch of NERL. A laboratory internal to the EPA, possibly the same lab that is used for marker development, or a laboratory under contract to the EPA could perform the genetic analysis.

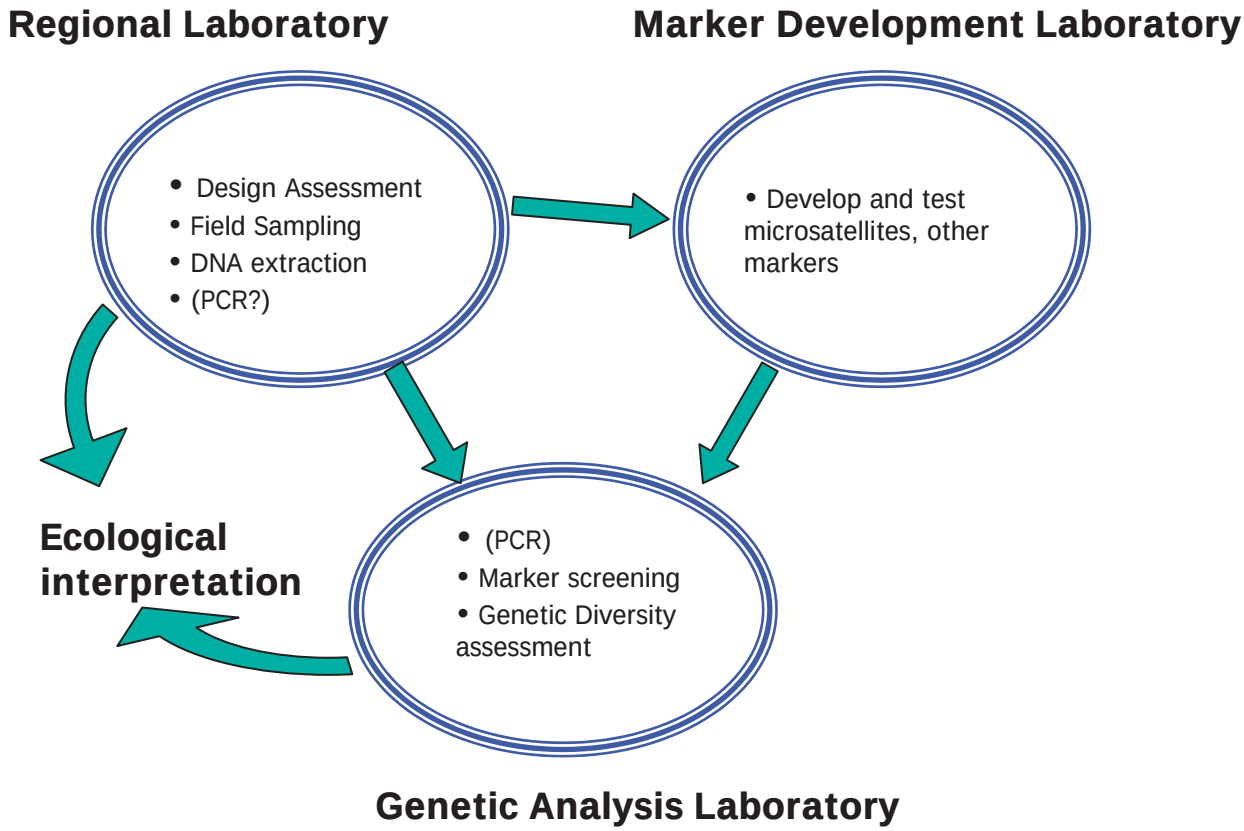

Figure 4-10. A model showing how three different labs, the regional field lab, a genetic analysis lab, and a marker development lab could interact to apply a genetic diversity indicator such as microsatellites or mitochondrial DNA sequences to an environmental assessment.